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Chapter 5

Prediction of methotrexate CNS distribution in different species – influence of disease conditions

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ABSTRACT

Children and adults with malignant diseases have a high risk of prevalence of the tumor in the central nervous system (CNS). As prophylaxis treatment methotrexate is often given. In order to monitor methotrexate exposure in the CNS, cerebrospinal fluid (CSF) concentrations are often measured. However, the question is in how far we can rely on CSF concentrations of methotrexate as appropriate surrogate for brain target site concentrations, especially under disease conditions.

In this study, we have investigated the spatial distribution of unbound methotrexate in healthy rat brain by parallel microdialysis, with or without inhibition of Mrp/Oat/Oatp-mediated active transport processes by a co-administration of probenecid. Specifically, we have focused on the relationship between brain extracellular fluid (brain_{ECF}) and CSF concentrations. The data were used to develop a systems-based pharmacokinetic (SBPK) brain distribution model for methotrexate. This model was subsequently applied on literature data on methotrexate brain distribution in other healthy and diseased rats (brain_{ECF}), healthy dogs (CSF) and diseased children (CSF) and adults (brain_{ECF} and CSF).

Important differences between brain_{ECF} and CSF kinetics were found, but we have found that inhibition of Mrp/Oat/Oatp-mediated active transport processes does not significantly influence the relationship between brain_{ECF} and CSF methotrexate concentrations.

The prediction of methotrexate data obtained in other healthy rats and dogs works reasonably well, provided that information on the different elimination routes, or the lack thereof, is included in the systems-based scaling approach. The prediction of data from diseased rats and humans, together with SBPK model-based simulations, indicates that disease conditions significantly affect brain distribution.

It is concluded that in parallel obtained data on unbound brain_{ECF}, CSF and plasma concentrations, under dynamic conditions, combined with advanced mathematical modeling is a most valid approach to develop SBPK models that allow revealing the mechanisms underlying the relationship between brain_{ECF} and CSF concentrations in health and disease.
INTRODUCTION

Methotrexate was introduced into the treatment of malignant diseases more than 50 years ago (Hertz et al., 1956). Today, many treatment protocols for malignant diseases, like acute lymphoblastic leukemia, have established the combination of high-dose methotrexate (to decrease the fraction of plasma protein binding), combined with leucovorin rescue (Djerassi et al., 1967; Moe and Holen, 2000). Before the use of central nervous system (CNS) prophylaxis, the CNS was the most frequently reported site of initial recurrence in children with acute lymphoblastic leukemia, accounting for up to 75% of cases (Bleyer and Poplack, 1985; Evans et al., 1970). However, in the treatment of acute lymphoblastic leukemia, prophylactic CNS therapy effectively reduced the rate of CNS relapses (Balis and Poplack, 1989; Blaney and Poplack, 1996, Clarke et al., 2003; Smith et al., 1996). Still, CNS recurrence remains a major limitation to achieving complete cure, accounting for 30–40% of recurrences in some pediatric clinical trials (Hutchinson et al., 2003; Lange et al., 2002).

The use of cranial irradiation for CNS prophylaxis is effective but associated with severe late effects (Clarke et al., 2003; Ochs and Mulhern, 1994). Today, the combination of high-dose methotrexate and intrathecal methotrexate, employed to reduce such treatment-related late effects, has successfully replaced cranial irradiation as CNS prophylaxis in most patients (about 80 to 90%) of acute lymphoblastic leukemia (Cáp et al., 1998; Pui et al., 2009). However, the intrathecal methotrexate procedure is susceptible to complications and stressful for the patient, especially for children (Keidan et al., 2005). Therefore, it has been suggested that the use of high-dose intravenous methotrexate alone could be sufficient for CNS prophylaxis (Niemann et al., 2010).

On the basis of in vitro testing, a methotrexate concentration of 0.45 µg/ml at the target site is commonly acknowledged as effective in killing tumor cells (Hryniuk and Bertino, 1969). With the CNS being the target site for CNS prophylaxis, it is the aim to have the appropriate methotrexate concentration in the brain extracellular fluid (brainECF). However, as blood-brain barrier (BBB) transport is highly restricted for methotrexate, plasma concentrations need to be far higher than the 0.45 µg/ml to be able to reach appropriate concentrations in the CNS. As a result, the exposure of the rest of the body to cytotoxic
concentrations is quite substantial (Chabner and Young, 1973; Ferreri et al., 2004).

The information on the relationship between plasma and CNS methotrexate concentrations appears to be inconsistent, with linear relationships reported by Borsi and Moe (1987), Jönsson et al. (2007), Millot et al. (1994), whereas Milano et al. (1990), Thyss et al. (1987) and Vassal et al. (1990) reported non-linear relationships. Therefore, CNS concentrations are often monitored for appropriate dose selection (Niemann et al., 2010). To that end, CSF concentrations are used, since brain_{ECF} concentrations are not readily measurable in humans. CSF concentrations are considered to be the best available surrogate (Fridén et al., 2009; Kalvass and Maurer, 2002; Liu et al., 2006; Liu et al., 2009; Maurer et al., 2005), with the assumption that CSF concentrations readily equilibrate with brain_{ECF} concentrations due to the lack of a physical barrier between these sites (Lee et al., 2001). However, due to qualitative and quantitative differences between processes that govern the pharmacokinetics (PK) of drugs in the brain_{ECF} versus CSF, a generally applicable relationship between the PK at these two sites does not exist and needs evaluation (Chapter 1; De Lange and Danhof, 2002; Lin, 2008; Shen et al., 2004).

We have previously shown that even for acetaminophen, investigated as model compound for passive transport into, within and out of the brain, differences exist between CSF and brain_{ECF} kinetics (Chapter 3). Furthermore, we have also shown that for quinidine, a model compound for P-gp mediated transport, differences exist between CSF and brain_{ECF} kinetics, which are very much dependent on P-gp functionality (Chapter 4). With methotrexate being a substrate for a wide variety of transporters that are all located at the BBB and BCSFB (including the reduced folate carrier 1 (RFC1) (Hinken et al., 2011), breast cancer resistance protein (BCRP) (Breedveld et al., 2007), multidrug resistance-associated proteins (MRP) 2, 3 and 4 (Vlaming et al., 2011), organic anion transporter (OAT) 1 and 3 (Takeda et al., 2002), and organic anion-transporting polypeptides (OATP) A (Badagnani et al., 2006) and OATP B (van de Steeg et al., 2009)), this could have major implications for the predictability of brain_{ECF} methotrexate concentrations on the basis of CSF concentrations. Consequently, to be able to know whether methotrexate brain_{ECF} concentrations can be adequately predicted by CSF concentrations, one should first understand
the mechanisms that determine the relationship between CSF concentrations and brain_{ECF} concentrations.

In this study we have used the parallel intracerebral microdialysis probes approach (striatum, lateral ventricle, and cisterna magna (Chapter 3)) in conjunction with parallel blood sampling, for continuous measurement and direct comparison of changes in concentrations in plasma, brain_{ECF} and CSF kinetics of methotrexate. To investigate the specific contribution of the various transporters, probenecid is co-administered as inhibitor of MRPs (Bakos et al., 2000), OATs (Sugiyama et al., 2001) and OATPs (Kis et al., 2013). Advanced mathematical modeling is applied to extrapolate the data to other conditions, and other species, including human.

**MATERIALS AND METHODS**

**Chemicals and solutions**

Methotrexate solution for injection (Emthexate PF) and isoflurane were obtained from Pharmachemie B.V. (Haarlem, the Netherlands). Methotrexate (powder), aminopterin and ammonium formate were from Sigma-Aldrich (Zwijndrecht, the Netherlands). Probenecid, 5% glucose and saline were obtained from the Leiden University Medical Centre pharmacy (Leiden, the Netherlands). Acetonitrile (HPLC-S grade), methanol (ULC-grade) and formic acid (ULC grade) were from Biosolve (Valkenswaard, the Netherlands). Ammonium hydroxide (25%), magnesium chloride, sodium acetate, sodium chloride, calcium chloride and perchloric acid, were obtained from Baker (Deventer, the Netherlands). Potassium chloride was from Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Microdialysis perfusion fluid was prepared as previously described (Chapter 3), containing 140.3 mM sodium, 2.7 mM potassium, 1.2 mM calcium, 1.0 mM magnesium and 147.7 mM chloride.
Animals

The study protocol was approved by the Animal Ethics Committee of Leiden University (UDEC nr. 10094). All animal procedures were performed in accordance with Dutch laws on animal experimentation. A total of 40 male Wistar WU rats (225-275 g, Charles River, Maastricht, the Netherlands) were randomly divided into two groups; the first group (n = 8) was used for the determination of the \textit{in vivo} microdialysis probe recovery; the second group (n = 32) was used for brain disposition experiments. This second group was further divided into four subgroups, designated for 40 or 80 mg/kg methotrexate without or with co-administration of probenecid (denoted as 40\textsuperscript{−}, 40\textsuperscript{+}, 80\textsuperscript{−} and 80\textsuperscript{+}, respectively).

After arrival, all animals were housed in groups for 5-7 days (Animal Facilities, Gorlaeus Laboratories, Leiden, the Netherlands), under standard environmental conditions (ambient temperature 21°C; humidity 60%; 12/12 h light/dark cycle, background noise, daily handling), with \textit{ad libitum} access to food (Laboratory chow, Hope Farms, Woerden, the Netherlands) and acidified water. Between surgery and experiments, the animals were kept individually in Makrolon type three cages for 7 days to recover from the surgical procedures.

Surgery

All surgical procedures were performed as described in Chapter 3. In short, cannulas were implanted in the left femoral artery and vein for blood sampling and drug administration, respectively. Both cannulas were subcutaneously led to the back of the head and fixated in the neck with a rubber ring. Subsequently, the animals were chronically instrumented with two CMA/12 microdialysis guides (CMA/Microdialysis AB, Stockholm, Sweden) in different combinations of striatum (ST), for sampling in brain\textsubscript{ECF}, and lateral ventricle (LV) and/or cisterna magna (CM) for sampling in CSF (ST+LV, ST+CM or LV+CM). For ST, the position of the microdialysis guide is: 1.0 mm anterior, 3.0 mm lateral, 3.4 mm ventral, relative to bregma. For LV, the position of the microdialysis guide is: 0.9 mm posterior, 1.6 mm lateral, 2.9 mm ventral, relative to the bregma. For CM, the position of the microdialysis guide is: 1.93 mm posterior, 3.15 mm lateral, 8.1 mm ventral, at an angle of 25° from the dorsoventral axis.
(towards anterior) and 18° lateral from the anteroposterior axis relative to lambda. The microdialysis guides were secured to the skull with 3 anchor screws and dental cement.

After the surgery the animals received 0.03 ml Temgesic® intramuscularly (Schering-Plough, Amstelvleen, the Netherlands) and 0.3 ml Ampicillan® (Alfasan B.V., Woerden, the Netherlands) subcutaneously. One day prior to the experiment, the microdialysis dummies were replaced by the microdialysis probes (CMA/12 Elite, Polyarylethersulfone membrane, molecular weight cut-off 20 kDa, CMA/Microdialysis AB, Stockholm, Sweden, with a semi-permeable membrane length of 4 mm for ST, and 1 mm for LV and CM).

**Experimental set-up**

All experiments were performed as described in Chapter 4, with some modifications. In short, the in vivo microdialysis probe recovery of methotrexate was determined on the basis of reverse dialysis (Ståhle et al., 1991). The microdialysis probes in striatum, lateral ventricle and cisterna magna were perfused with different concentrations of methotrexate (50, 200 and 1000 ng/ml) in perfusion fluid. To evaluate the potential effect of co-administration of probenecid on the in vivo recovery of methotrexate, several animals received an intravenous infusion of 150 mg/kg probenecid in 5% NaHCO₃ in saline (150 µl/min/kg for a period of 10 minutes) with an automated pump (Pump 22 Multiple Syringe Pump, Harvard Apparatus, Holliston, USA) 30 minutes prior to the start of the reverse dialysis experiment. Control animals received an intravenous infusion of vehicle (150 µl/min/kg, for a period of 10 minutes).

The in vivo recovery is defined as the ratio of the concentration difference between the dialysate (C_dial) and perfusion fluid (C_in) over the concentration in the perfusion fluid (equation 1) (Scheller and Kolb, 1991).

\[
\text{in vivo recovery} = \frac{C_{\text{in}} - C_{\text{dial}}}{C_{\text{in}}} \quad \text{(1)}
\]

For the brain disposition experiments, the rats first received an intravenous infusion of 150 mg/kg probenecid in 5% NaHCO₃ in saline or vehicle (150 µl/min/kg, for a period of 10 minutes) 30 minutes prior to the administration of 40 or 80 mg/kg methotrexate in saline (200 µl/min/kg for a period of 10
The start and duration of the infusion was corrected for internal volume of the tubing so that infusion started at \( t = 0 \) min. 10 min interval samples were collected between \( t = -1 \) h and \( t = 5 \) h. After weighing the microdialysis vials they were stored at \(-80^\circ C\) before analysis.

For the determination of methotrexate plasma concentrations, blood samples of 100 μl were taken, in parallel to the microdialysate samples, from the arterial cannula at \( t = -5 \) (blank), 2, 7, 9, 10, 12, 17, 30, 90, 180, and 300 min. All blood samples were temporarily stored in heparin (10 IU) coated Eppendorf cups before being centrifuged for 15 min at 5000 rpm. The plasma was then pipetted into clean Eppendorf cups and stored at \(-20^\circ C\) before analysis.

At the end of the experiments the animals were sacrificed with an overdose of Nembutal (Ceva Sante Animale, Libourne, France).

**Plasma protein binding**

For the determination of plasma protein binding of methotrexate, plasma samples of different time points were pooled (combining \( t = 2 \) and \( t = 7 \); \( t = 9 \) and \( t = 10 \); \( t = 12 \) and \( t = 17 \); \( t = 30 \) and \( t = 90 \); \( t = 180 \) and \( t = 300 \)) to span the entire concentration range. Plasma protein binding was determined with Centrifree® ultrafiltration devices (Millipore BV, Etten-Leur, the Netherlands). All procedures were performed according to the user’s manual. The ultrafiltrate was diluted 10 times with saline before the analysis.

**Concentration analysis**

Methotrexate concentrations in plasma, plasma ultrafiltrate and microdialysate were quantified using an on-line solid phase extraction (SPE) with liquid chromatography-tandem mass spectrometry (LC/MS/MS) system, based on previous work by Rule *et al.* (2001) and Guo *et al.* (2007).

To 20 μl of plasma and plasma ultrafiltrate, 20 μl internal standard (10 μg/ml aminopterin) was added. After mixing with 40 μl of 6% perchloric acid and centrifugation for 10 minutes at 10000 g, 60 μl of the supernatant was thoroughly mixed with 40 μl of 1 M sodium acetate. 20 μl was then injected on the SPE column.
To 15 µl of the microdialysate samples, 15 µl of the internal standard (250 ng/ml aminopterin) was added. After mixing, 10 µl was injected on the SPE column.

HySphere™ SPE cartridges and a cartridge holder from Spark (Emmen, the Netherlands) were used in combination with the divert/inject valve of the mass spectrometer to prevent salt entering the mass spectrometer. After flushing under acidic conditions, the SPE was switched onto the LC system and methotrexate and its internal standard were eluted from the SPE to the LC column. For plasma precipitates, C-8 HD SE cartridges were used, while for microdialysate samples C-18 cartridges were used. After injection of the sample on the SPE column, salts from either precipitated plasma or microdialysate samples were flushed to waste before analysis on the HPLC column. A quaternary gradient HPLC pump (P580) from Dionex (Breda, the Netherlands) was used for the on-line SPE method, while a Surveyor pump (Thermo Scientific, Breda, the Netherlands) served as delivery unit for the HPLC column.

The VisionHT® C-18B column (Grace Alltech, Breda, the Netherlands) was thermostatted at 37°C. The mobile phase consisted of 21% methanol and 79% lab water, derived from a PURELAB Ultra system (Veolia Water Solutions, Ede, the Netherlands), containing 0.2% formic acid and 1 mM ammonium formate. After isocratic elution of the peaks of methotrexate and internal standard, the column was flushed with 90% methanol and 10% lab water containing 0.2% formic acid and 1mM ammonium formate 7–9.5 minutes after injection.

Sample analysis was performed on a Finnigan TSQ Quantum Ultra Mass Spectrometer System (Thermo Scientific, Breda, the Netherlands). Electrospray ionization was used in the positive mode at 3500V. Methotrexate was quantified using selected reaction monitoring (SRM) with the transition 455–308 (m/z). The internal standard, aminopterin, had a SRM transition of 441–294 (m/z). The collision energy used was 18 V, scan width was set at 0.7 m/z and both Q1 and Q3 were set to 0.70 full width at half maximum (FWHM). The scan time was 0.11 seconds. Argon served as the collision gas.
Pharmacokinetic data analysis

All plasma concentrations were converted to unbound plasma concentrations, by correction for plasma protein binding. All microdialysate concentrations from striatum, lateral ventricle and cisterna magna were converted into brain_{ECF} concentrations (C_{ECF}) or CSF concentrations (C_{CSF}) by division of the dialysate concentrations by the average \textit{in vivo} recovery as determined for each microdialysis probe location (equation 2).

\[
C_{ECF} \text{ or } C_{CSF} = \frac{C_{dial}}{\text{in vivo recovery}} 
\]  

(2)

Areas under the curve from t=0 to t=300 min (AUC{0-300}) were calculated by the trapezoidal rule and tested for differences by single factor ANOVA. The population PK models were developed and fitted to the data by means of non-linear mixed-effects modeling using the NONMEM software package (version 6.2, Icon Development Solutions, Ellicott City, Maryland, USA) and analyzed using the statistical software package S-Plus® for Windows (version 6.2 Professional, Insightful Corp., Seattle, USA).

The pharmacokinetic model for methotrexate plasma and brain concentrations was based on the systems-based PK (SBPK) approach we have previously applied to investigate the exchange between brain_{ECF} and CSF of acetaminophen (Chapter 3) and quinidine (Chapter 4). For this approach, the volumes of the different brain compartments were fixed to their physiological volumes. The rat brain intracellular space and brain_{ECF} volume were assumed to be 1.44 ml (Thorne \textit{et al.}, 2004) and 290 µl (Cserr \textit{et al.}, 1981), respectively. With a total CSF volume of 300 µl in the rat (Bass and Lundborg, 1973), the volumes of the lateral ventricles, third and fourth ventricles, cisterna magna and subarachnoid space were assumed to be 50 µl (Condon \textit{et al.}, 1986; Kohn \textit{et al.}, 1991), 50 µl (Levinger, 1971), 17 µl (Adam and Greenberg, 1978; Robertson, 1949) and 180 µl (Bass and Lundborg, 1973; Levinger, 1971), respectively. The intra-brain distribution was restricted by the physiological flow paths of brain_{ECF}, in which brain_{ECF} flows towards the CSF compartments at a rate of 0.2 µl/min (Abbott, 2004; Cserr \textit{et al.}, 1981), and CSF flows from lateral ventricle, through the third and fourth ventricle, to the cisterna magna and subsequently to
the subarachnoid space (cranial and spinal) and back into blood at a rate of 2.2 μl/min (Cserr, 1965).

Structural model selections for both the blood and brain PK model were based on the likelihood ratio test ($p < 0.01$), diagnostic plots (observed concentrations vs. individual and population predicted concentrations, weighted residuals vs. predicted time and concentrations), parameter correlations and precision in parameter estimates. The inter-animal variability in pharmacokinetic parameters was assumed to be log normally distributed. The residual error, which accounts for unexplained variability (e.g. measurement and experimental error and model-misspecification), was best described with a proportional error model.

The validity of the pharmacokinetic models was investigated by means of a visual predictive check. (Cox et al., 1999; Duffull and Aarons, 2000; Yano et al., 2001) Using the final PK parameter estimates, 1000 curves were simulated. Subsequently, the median and the 5th and 95th percentile of the predicted concentrations were calculated, which represent the 90% prediction interval. These were then compared with the observations.

In order to test the ruggedness of the model and estimate the precision of the parameters n=100 non-parametric (case resampling) bootstraps were performed. To create the bootstrapped datasets, specific rat data (plasma and microdialysate concentrations) were removed randomly from the datasets and replaced with randomly selected rat data from the complete original dataset. Each of these permutations of the original dataset was fitted with the final model determined based on the original dataset. This results in a series of model fits, each with its own set of parameters. These results were displayed graphically and the descriptive statistics of the parameters were compared to parameter estimates of the final model. Only bootstrap runs that successfully minimized were used in this analysis.

**Systems-based scaling**

The SBPK model was first used to predict the methotrexate plasma and brain$_{ECF}$ concentration-time profiles of brain tumor-bearing rats (De Lange et al., 1995), to investigate the impact of disease-status on the kinetics of methotrexate. Next, the parameters of the rat SBPK were extrapolated to healthy dogs (Neuwelt et
al., 1985), to investigate the validity of using this approach for interspecies scaling. To do so, the elimination clearance was first divided into renal clearance and hepatic clearance, with renal clearance assumed to be identical to the glomerular filtration rate (BrcaKova et al., 2009) and the remaining clearance assigned to hepatic clearance. The hepatic clearance was then scaled to the number of hepatocytes in rat or dog liver. Next, the transfer clearances between plasma and the different peripheral and brain compartments were scaled based on

\[ CL_{PL-PE} = A_{PE} \times (V_{PE})^{0.67} \]  
(3)

\[ CL_{PL-BR} = A_{BR} \times (V_{BR})^{0.67} \]  
(4)

In which \( A_{PE} \) and \( A_{BR} \) are scaling coefficients for periphery and brain, respectively, and both are estimated on the basis of the rat data (Hosseini-Yeganeh and McLachlan, 2002). \( V_{PE} \) represents the physiological volume of the (lumped) peripheral tissues for rapid (including muscle, kidney, intestine and liver) and slow equilibration (including adipose, skin, heart, bone and remaining tissue). \( V_{BR} \) represents the physiological volume of the different brain compartments. The scaling factor of 0.67 is based on the permeability surface area of the different brain compartments, which is related to the tissue weight (Kawai et al., 1994). The physiological parameters of the rat SBPK model were changed accordingly.

Finally, the parameters of the rat SBPK were extrapolated to the human setting, which included adults and children with different disease-states, using the same approach as applied for extrapolation to dogs.

**RESULTS**

All results are presented as average values ± standard error of the mean, unless stated otherwise.
Methotrexate pharmacokinetics

The average unbound plasma (plasma\textsubscript{u}), brain\textsubscript{ECF}, lateral ventricle (CSF\textsubscript{LV}) and cisterna magna (CSF\textsubscript{CM}) methotrexate concentrations following the 40 and 80 mg/kg dose with or without co-administration of probenecid are shown in figure 1. Plasma protein binding of methotrexate was linear at an extent of 55.2 ± 7.7%. It was not affected by co-administration of probenecid. The co-administration of probenecid slightly altered the distribution phase for both the 40 and 80 mg/kg dose of methotrexate. Data obtained by microdialysis from the brain\textsubscript{ECF}, CSF\textsubscript{LV} and CSF\textsubscript{CM} were corrected for in vivo recovery. The average in vivo recoveries for the methotrexate concentrations in striatum, lateral ventricle and cisterna magna probes were influenced by co-administration of probenecid and were determined to be 22.1 ± 2.0%, 28.1 ± 2.9% and 35.9 ± 2.5%, for the control group and 7.1 ± 0.9%, 16.9 ± 1.7% and 21.6 ± 5.6% for the probenecid group, respectively.

It can be seen that a higher dose of methotrexate leads to higher methotrexate concentrations in all brain compartments, but not to the same extent. Probenecid increased methotrexate concentrations significantly (p < 0.01) in all brain compartments. The effect of probenecid was dependent on the methotrexate dose; at the higher dose of methotrexate, the increase in methotrexate concentrations was more profound for brain\textsubscript{ECF} and CSF\textsubscript{LV}, as can be seen by the average unbound brain (brain\textsubscript{u})-to-plasma\textsubscript{u} AUC\textsubscript{0-300} ratios (table 1). However, the dose-dependency was not significant. The relationship between brain\textsubscript{ECF}-to-CSF concentration ratios was not significantly affected by probenecid co-administration, and was on average 7.7 ± 3.7 (table 2).

Table 1. Brain\textsubscript{u}-to-plasma\textsubscript{u} AUC\textsubscript{0-360} ratios for brain\textsubscript{ECF}, CSF\textsubscript{LV} and CSF\textsubscript{CM} for the 40 mg/kg and 80 mg/kg dose without (-) and with (+) co-administration of probenecid

<table>
<thead>
<tr>
<th>Brain\textsubscript{u}-to-plasma\textsubscript{u} AUC\textsubscript{0-300} ratios</th>
<th>40’</th>
<th>40‡</th>
<th>80’</th>
<th>80‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain\textsubscript{ECF}</td>
<td>2.5 ± 1.7%</td>
<td>5.9 ± 1.1%</td>
<td>2.5 ± 0.3%</td>
<td>9.8 ± 3.3%</td>
</tr>
<tr>
<td>CSF\textsubscript{LV}</td>
<td>0.6 ± 0.1%</td>
<td>1.6 ± 1.0%</td>
<td>0.5 ± 0.3%</td>
<td>3.5 ± 0.4%</td>
</tr>
<tr>
<td>CSF\textsubscript{CM}</td>
<td>0.4 ± 0.1%</td>
<td>0.8 ± 0.1%</td>
<td>0.7 ± 0.4%</td>
<td>0.9 ± 0.5%</td>
</tr>
</tbody>
</table>

\* Significantly (p < 0.05) different from the group without co-administration of probenecid

\‡ Significantly (p < 0.05) different from the CSF-to-plasma\textsubscript{u} AUC\textsubscript{0-300} ratios
Figure 1. Average (geometric mean ± S.E.M.) unbound methotrexate concentration-time profiles following intravenous administration of methotrexate, with (+) or without (-) co-administration of probenecid (150 mg/kg). (A) 40 mg/kg methotrexate dose: for plasma (n = 7 (-) and 6 (+)), brain$_{ECF}$ (n = 5 (-) and 4 (+)), CSF$_{LV}$ (n = 4 (-) and 3 (+)) and CSF$_{CM}$ (n = 3 (-) and 4 (+)). (B) 80 mg/kg methotrexate dose. Plasma (n = 7 (-) and 7 (+)), brain$_{ECF}$ (n = 4 (-) and 5 (+)), CSF$_{LV}$ (n = 5 (-) and 4 (+)) and CSF$_{CM}$ (n = 6 (-) and 3 (+))

Table 2. Brain$_{ECF}$-to-CSF concentration ratios for the 40 mg/kg and 80 mg/kg dose without (-) and with (+) co-administration of probenecid. (No significant differences were found).

<table>
<thead>
<tr>
<th>Brain$_{ECF}$-to-CSF concentration ratios</th>
<th>40$^-$</th>
<th>40$^+$</th>
<th>80$^-$</th>
<th>80$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain$<em>{ECF}$-to-CSF$</em>{LV}$</td>
<td>9.6 ± 5.0</td>
<td>6.0 ± 2.9</td>
<td>11.6 ± 7.0</td>
<td>5.2 ± 2.4</td>
</tr>
<tr>
<td>Brain$<em>{ECF}$-to-CSF$</em>{CM}$</td>
<td>11.5 ± 5.5</td>
<td>8.3 ± 1.6</td>
<td>6.0 ± 2.3</td>
<td>12.7 ± 3.0</td>
</tr>
<tr>
<td>Brain$<em>{ECF}$-to-CSF$</em>{average}$</td>
<td>10.2 ± 4.8</td>
<td>6.8 ± 2.1</td>
<td>7.6 ± 3.3</td>
<td>7.2 ± 2.7</td>
</tr>
</tbody>
</table>

Systems-based modeling approach

As it was our goal to investigate the relationship between brain$_{ECF}$ and CSF pharmacokinetics, we have applied a SBPK modeling approach like we did previously for analysis of regional brain distribution of acetaminophen (Chapter 3) and quinidine (Chapter 4). To adequately describe CSF physiology, we have used four CSF compartments that represent the CSF$_{LV}$, the combined third and fourth ventricle (CSF$_{TFV}$), the CSF$_{CM}$ and the subarachnoid space (CSF$_{SAS}$). Since we have no measurements of the concentrations in the third and fourth ventricle, the transfer clearance between plasma and third and fourth ventricle was assumed to be equal to the transfer clearance between plasma and lateral ventricle.
Distinction between passive and active transport clearances - The active component of the different transfer clearances between plasma and the brain compartments was determined by comparing the parameter estimations for the rats that did to those rats that did not receive the co-administration of probenecid. Even though methotrexate is transported by a wide variety of transporters, the co-administration of the Mrp-, Oat- and Oatp-inhibitor probenecid allowed us to investigate the impact of combined Mrp-, Oat- and Oatp-mediated transport. The active component of the transfer clearances between plasma and the different brain compartments was incorporated into the model as previously described by Syvänen et al. (2006):

\[ CL_{PL- BR} = CL_{PL- BR, p} - CL_{PL- BR, a} \]  
\[ CL_{BR- PL} = CL_{BR- PL, p} + CL_{BR- PL, a} \]

Where the subscript ‘p’ denotes passive transport and ‘a’ denotes active transport. For the animals that received a co-administration of probenecid, both \( CL_{PL- BR, a} \) and \( CL_{BR- PL, a} \) were considered to be 0.

Modeling Mrp-, Oat- and Oatp-mediated transport - Both Mrps, Oats, as well as Oatps have been well described as efflux transporters at the BBB and BCSFB (Graff and Pollack, 2004). However, the mechanism by which Mrps, Oats and Oatps can exert their effect could be by reducing the transfer clearance from plasma to the brain compartments (i.e. influx hindrance; equation 5) or by increasing the transfer clearance from the brain compartments to plasma (i.e. efflux enhancement; equation 6) or both. The data were best described by the model with Mrp, Oat and Oatp functioning solely by influx hindrance. Interestingly, the observation that in vivo probe recovery of methotrexate was affected by probenecid indicates that methotrexate is transported by Mrps, Oats and Oatps via the efflux enhancement mechanism (Syvänen et al., 2006). However, estimation of the active components when Mrp, Oat and Oatp were considered to function by efflux enhancement resulted in too large coefficients of variation. Also, the estimation of the active component in the transfer between plasma and cisterna magna resulted in too large coefficients of variation and was therefore assumed to be absent.
Modeling methotrexate concentration-dependent Mrp-, Oat- or Oatp-mediated transport - Since Mrp-, Oat- or Oatp-mediated transport is an active (saturable) process we have also tried to identify the maximal transport rate (\(T_m\)) and the blood- or brain concentration for half-maximal transport (\(K_m\)) as follows:

\[
CL_{PL-\overline{BR},a} = \frac{T_{m,PL-\overline{BR}}}{K_{m,PL-\overline{BR}} + C_{PL,u}} \tag{7}
\]

\[
CL_{BR-\overline{PL},a} = \frac{T_{m,BR-\overline{PL}}}{K_{m,BR-\overline{PL}} + C_{BR}} \tag{8}
\]

Where \(C_{PL,u}\) is the unbound plasma concentration and \(C_{BR}\) is the concentration in one of the brain compartments. The parameter estimations of \(T_m\) and \(K_m\) resulted in high values for both \(T_m\) and \(K_m\) (results not shown), indicating that the plasma and brain concentrations in this study are not sufficiently high for saturating Mrp-, Oat- or Oatp-mediated transport. The parameter estimations of \(T_m\) and \(K_m\) also resulted in too large coefficients of variation. Thus, our data were insufficient to determine the values of these parameters. Therefore, Mrp-, Oat- or Oatp-mediated transport had to be incorporated by means of a single active transport clearance value, rather than by \(T_m\) and \(K_m\).

Final SBPK model - The final SBPK model is shown in figure 2. The differential equations of this model can be found in the appendix. The final estimation of the PK parameters is summarized in table 3. Interestingly, no active component on the elimination clearance could be identified, whereas this was the case for the compartmental pharmacokinetic analysis of the plasma concentrations only (results not shown).

According to Brcakoa et al. (2009), the rate of renal clearance of unbound methotrexate in rats equals the rate of renal creatinine clearance, which is a measure of the glomerular filtration rate (GFR; 1.4 ml/min in the rat (Atherton, 1983)). Consequently, the remaining methotrexate clearance is hepatically. Based on our results, the rate of hepatic clearance is therefore 7.35 ml/min in the rat. With a total hepatic blood flow of approximately 11.8 ml/min (Davies and Morris, 1993), this results in a hepatic extraction ratio of 62%, which is comparable to the 53% reported previously by Kates and Tozer (1976).
Prediction of methotrexate CNS distribution in different species – influence of disease conditions

Figure 2. Diagram of the SBPK model that was used to describe the intra-brain distribution of methotrexate in the rat. $CL_E$ is the elimination clearance from plasma. Further, for transfer clearances between compartments ($CL_{from \; comp-to \; comp}$), denotations of the compartments are: $PL =$ plasma; $PER_{rapid} =$ rapidly equilibrating periphery; $PER_{slow} =$ slowly equilibrating periphery; $ECF =$ brain ECF; $LV =$ lateral ventricle; $TFV =$ third and fourth ventricle; $CM =$ cisterna magna and $SAS =$ subarachnoid space. $Q_{ECF}$ is the flow rate of brain ECF, $Q_{CSF}$ is the flow rate of CSF. The subscript ‘p’ denotes passive transport and ‘a’ denotes active transport.
Chapter 5

The visual predictive check of the final model is given in figure 3. It can be seen that the final model describes the data very well within the 90% prediction interval, and also can cope with the large inter-individual variation in brain concentrations.

![Figure 3](image)

*Figure 3. The visual predictive check of the final SBPK model. The dots represent the individual data points and the gray area represents the 90% prediction confidence interval. The different boxes represent the plasma$_u$, brain$_{ECF}$, CSF$_{LV}$ and CSF$_{CM}$ data*

**Systems-based scaling**

The physiological and PK parameters of the rat, dog and human children and adults that were used for the extrapolation are given in table 3.
Table 3. Final estimation of the rat PK parameters of methotrexate for the SBPK model (± standard error) and predicted dog and human parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rat value</th>
<th>Dog value (20-25 kg)</th>
<th>Human child value (9 year old, 30 kg bodyweight)</th>
<th>Human adult value</th>
</tr>
</thead>
<tbody>
<tr>
<td>f_u,p</td>
<td>44.8 ± 7.7 %</td>
<td>60% (^{13})</td>
<td>67.7% (^{19})</td>
<td>67.7% (^{19})</td>
</tr>
<tr>
<td>CL_E,p</td>
<td>8.75 ± 0.85 ml/min</td>
<td>88.2 ml/min ((\text{based on } 1.1 \times \text{GFR})^{13})</td>
<td>163.2 ml/min ((\text{based on } 2 \times \text{GFR})^{20})</td>
<td>250 ml/min ((\text{based on } 2 \times \text{GFR})^{20})</td>
</tr>
<tr>
<td>GFR</td>
<td>1.4 ml/min (^{1})</td>
<td>80.2 ml/min (^{14})</td>
<td>81.6 ml/min (^{27})</td>
<td>125 ml/min (^{6})</td>
</tr>
<tr>
<td>CL_{PL-E CF,a}</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>CL_{PL-LV,a}</td>
<td>0.09 ± 0.03 µl/min</td>
<td>1.64 µl/min</td>
<td>4.17 µl/min</td>
<td>5.39 µl/min</td>
</tr>
<tr>
<td>Q_{ECF}</td>
<td>0.2 µl/min (^2,) (^3)</td>
<td>10.5 µl/min ((\text{based on } 0.11 \mu l/min/g \text{brain})^{3})</td>
<td>0.1 µl/min ((\text{based on } 50% \text{ of } Q_{CSF})^{22})</td>
<td>0.175 ml/min (^{22})</td>
</tr>
<tr>
<td>Q_{CSF}</td>
<td>2.2 µl/min (^4)</td>
<td>63 µl/min (^{13})</td>
<td>0.2 µl/min (^{24})</td>
<td>0.4 ml/min (^{29})</td>
</tr>
<tr>
<td>V_{PL}</td>
<td>10.6 ml (^3)</td>
<td>1112 ml (^{15})</td>
<td>1600 ml (^{24})</td>
<td>2900 ml (^{29})</td>
</tr>
<tr>
<td>V_{PER,rapid}</td>
<td>49.7% of bodyweight (^6)</td>
<td>64.1% of bodyweight (^6)</td>
<td>36.1% of bodyweight (^25)</td>
<td>35.0% of bodyweight (^25)</td>
</tr>
<tr>
<td>Parameter</td>
<td>Value</td>
<td>Source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-------</td>
<td>--------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{PER,slow}} )</td>
<td>45.5% of bodyweight</td>
<td>( V_{\text{ECF}} )</td>
<td>290 µl</td>
<td>19 ml (based on 95.5 gr brain)</td>
</tr>
<tr>
<td>( V_{\text{ECF}} )</td>
<td>290 µl</td>
<td>19 ml (based on 95.5 gr brain)</td>
<td>270 ml (based on 1350 gr brain)</td>
<td>280 ml (based on 1400 gr brain)</td>
</tr>
<tr>
<td>( V_{\text{CSF,total}} )</td>
<td>300 µl</td>
<td>15.6 ml</td>
<td>90 ml</td>
<td>140 ml</td>
</tr>
<tr>
<td>( V_{\text{LV}} )</td>
<td>50 µl</td>
<td>3.8 ml (24% of total CSF volume)</td>
<td>15.3 ml (17% of total CSF volume)</td>
<td>22.5 ml</td>
</tr>
<tr>
<td>( V_{\text{TFV}} )</td>
<td>50 µl</td>
<td>1.3 ml (8% of total CSF volume)</td>
<td>15.3 ml (17% of total CSF volume)</td>
<td>22.5 ml</td>
</tr>
<tr>
<td>( V_{\text{CM}} )</td>
<td>17 µl</td>
<td>0.9 ml (6% of total CSF volume (human value))</td>
<td>5.1 ml (5.7% of total CSF volume)</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>( V_{\text{SAS}} )</td>
<td>180 µl</td>
<td>9.0 ml (60% of total CSF volume (human value))</td>
<td>54 ml (60% of total CSF volume)</td>
<td>90 ml</td>
</tr>
</tbody>
</table>

Parameter values in italic are derived from literature. \( F_{u,p} \) is the fraction unbound in plasma, \( \text{CLE} \) is the elimination clearance from plasma. For transfer clearances between compartments (\( \text{CL}_{\text{from comp-to comp}} \)), denotations of the compartments are: \( \text{PL} = \text{plasma}; \text{PER,rapid} = \text{rapidly equilibrating peripheral tissues}; \text{PER,slow} = \text{slowly equilibrating peripheral tissues}; \text{ECF} = \text{brainECF}; \text{LV} = \text{lateral ventricle}; \text{TFV} = \text{third and fourth ventricle}; \text{CM} = \text{cisterna magna}; \text{and SAS} = \text{subarachnoid space}. \( V \) is the physiological volume. \( \eta_i \) = inter-individual variability of parameter \( i \); \( \varepsilon_j \) = residual error on concentrations in compartment \( j \). The additional subscripts ‘p’ and ‘a’ denote passive and active transport, respectively. N.A. implicates that the parameter is not available in the specific model.

Extrapolation to other healthy and to diseased rats

We have applied the final SBPK model to investigate the impact of disease-status on the PK of methotrexate in plasma_{u} and brain_{ECF} of brain tumor-bearing rats, compared to healthy control rats (De Lange et al., 1995). By using the same PK parameter values that were estimated based on our data (table 3), there appears to be a small underestimation of the elimination from plasma as well as a small underestimation of the initial brain_{ECF} concentrations for the control rats (figure 4A) (but both plasma_{u} and brain_{ECF} data are in general still within the range of 5-95% of the model prediction of the data). However, for the group of rats with measurement of plasma and ipsilateral brain_{ECF} concentrations at 11 days post-tumor implantation, the concentration-time profiles in brain_{ECF} in brain tumor were substantially higher than was the case for the healthy situation (figure 4B). Presence and size of tumor were determined histologically after the end of the experiment. Simulations indicated that these higher brain_{ECF} concentrations are most likely caused by an increased plasma-to-brain_{ECF} clearance rate in brain tumor conditions (results not shown).

![Figure 4. Observed and SBPK model predicted methotrexate plasma_{u} and brain_{ECF} concentrations in (A) control rats (plasma_{u}, n=5; brain_{ECF}, n=6); (B) tumor implanted rats with ipsilateral brain_{ECF} measurement after 11 days (plasma_{u}, n=6; brain_{ECF}, n=6) (De Lange et al., 1995). Note that the two lowest concentration-time profiles in (B) represent animals in which no tumor was present on subsequent histological examinations. The dots represent the individual data points and the gray area represents the 90% prediction confidence interval. The different boxes represent the plasma_{u} and brain_{ECF} data](image-url)
Extrapolation to healthy dogs

We have also tried to predict the plasma_u and CSF kinetics of methotrexate in healthy dogs by systems-based scaling of our rat data. Predictions were then compared to literature data presented by Neuwelt et al. (1985). Plasma concentrations were first corrected for the level of protein binding, which was assumed to be 40% (Henderson et al., 1965). The rate of renal clearance was assumed to equal the glomerular filtration rate (80.2 ml/min (Von Hendy-Willson and Pressler, 2011)). Systems-based scaling of our data initially resulted in a 10-to-100-fold underestimation of dog plasma_u and CSF_{CM} concentrations, whereas the CSF_{CM}-to-plasma_u concentration ratio was predicted correctly. The underestimation of the plasma_u and CSF_{CM} concentrations was primarily caused by an overestimation of the plasma elimination clearance. As the number of hepatocytes per gram liver in dogs is twice as much compared to rats (Bayliss et al., 1999), the rate of hepatic clearance was scaled to the liver weight and then multiplied by 2. With an assumed liver weight of 720 g in the dog (Davies and Morris, 1993), the rate of hepatic clearance was estimated to be 1094 ml/min. However, in the rat, methotrexate is eliminated both via the kidneys as well as the liver, whereas in dogs methotrexate is eliminated primarily via the kidneys (Henderson et al., 1965). This indicates that the estimation of the hepatic clearance rate in dogs was much too high. For healthy dogs, with a hepatic clearance being approximately 10% of the renal clearance (Henderson et al., 1965), using the physiological and adapted PK parameters presented in table 3, the prediction was much improved and resulted in a slight underestimation of plasma_u and CSF_{CM} concentrations (figure 5). Yet the CSF_{CM}-to-plasma_u concentration ratio was still predicted correctly.
Figure 5. Observed and SBPK model predicted methotrexate plasma and CSF concentration-time profiles in (healthy) dogs (plasma, n=12; CSF, n=4). The dots represent the average data points as could be obtained from Neuwelt et al. (1985) and the gray area represents the 90% prediction confidence interval. The different boxes represent the plasma and CSF data. The hepatic clearance was assumed to be 10% of the renal clearance for the final SBPK predictions.

**Extrapolation to diseased human adults and children**

The only available human brain concentration-time profiles were derived from Blakeley et al. (2009), from 2 adult patients with supratentorial glioma. Other data included human plasma and CSF LV concentration-time profiles in adults with meningeal leukemia or meningeal carcinomatosis (n = 21, Shapiro et al., 1975), primary tumor (n = 16, Glantz et al., 1998), or only plasma concentration-time profiles in adults with small-cell lung carcinoma (n = 9, Creaven et al., 1976), various neoplastic diseases (n = 8, Bore et al., 1987) or rheumatoid arthritis (n = 56, Herman et al., 1989; Stewart et al., 1990). Also included were plasma and CSF concentration-time profiles from children with medulloblastoma or ependymoblastoma (n = 4, Chatelut et al., 1991), plasma and CSF concentration-time profiles from children with non-Hodgkin’s lymphoma (n = 29, Vassal et al., 1990), or only plasma concentration-time profiles from children with acute lymphoblastic leukemia (n = 49, Aumente et al., 2006) or osteosarcoma (n = 14, Colom et al., 2009). For constructing the adult and child plasma, CSF LV, CSF CM and CSF SAS dataset, either the individual or average data points from the different references were used, when available. Otherwise, simulations were performed based on the PK parameters presented in the different references.
All plasma concentrations were first corrected for the level of protein binding, which was assumed to be 32% for high dose methotrexate (Skibińska et al., 1990). Systems-based scaling of our data initially resulted in a 100-fold underestimation of human plasma, brainECF and CSF concentrations for both adults and children (results not shown). Again, the underestimation of the plasma, brainECF and CSF concentrations was primarily caused by an overestimation of the elimination clearance. For the systems-based scaling of the elimination clearance of methotrexate it was assumed that the rate of human renal clearance equals the glomerular filtration rate (125 ml/min in adults (Davies and Morris, 1993) and approximately 80 ml/min in 9 year-old children (the average age of the available datasets)). As the number of hepatocytes per gram liver is equal between rats and humans (Bayliss et al., 1999), the rate of hepatic clearance is scaled to the liver weight. With an assumed liver weight of 10 g in the rat (Davies and Morris, 1993), 780 g in 9 year old children and 1820 g in adults (ICRP, 2002), the rate of hepatic clearance is estimated to be 593 ml/min and 1380 ml/min in 9 years old children and adults, respectively. However, due to the extensive enterohepatic circulation of methotrexate in humans, the hepatic elimination rate is effectively reduced to the same level as the renal elimination rate (Hendel and Brodthagen, 1984). So, when taking into account that the hepatic clearance rate of methotrexate in humans is much lower than the extrapolated value, i.e. equal to the renal clearance rate, the predicted human plasma concentrations are comparable to the observed concentrations (figure 6). However, even though the human plasma, concentrations can be predicted reasonably well with systems-based scaling of our rat data and adaption of the hepatic clearance rate to those reported for human (table 3), this approach still results in an up to 10-fold underestimation of brainECF, CSF_CM and CSF_LV concentrations in both children and adults, respectively. Scaling of the blood-to-brain clearance values on the basis of the total surface area of the BBB (150 cm² in rats (Gjedde, 1981; Keep and Jones, 1990a); 200,000 cm² in human adults (Pardridge, 2002)) and BCSFB (75 cm² in rats (Keep and Jones, 1990b), 2000-100,000 cm² in human adults (Dohrmann, 1970)), rather than to the tissue weight to the power 0.67, did not improve the predictions (results not shown).

BrainECF and CSF_LV concentrations in adults, as shown in figure 6a, were not obtained in parallel, but from different subjects, also having other diseases. However, these are the only data available to make a comparison between these
two unbound brain concentrations. Both $\text{brain}_{\text{ECF}}$ and $\text{CSF}_{\text{LV}}$ concentrations are higher than predicted by the SBPK model, while the observed $\text{brain}_{\text{ECF}}$-to-$\text{CSF}_{\text{LV}}$ concentration ratio is in accordance with the predicted $\text{brain}_{\text{ECF}}$-to-$\text{CSF}_{\text{LV}}$ concentration ratio. It therefore seems that the plasma-to-brain concentration ratio is increased by disease conditions. Specifically, simulations indicated that the $\text{brain}_{\text{ECF}}$-to-CSF concentration ratio in diseased adults and children is equal to 2.6, which is approximately 3-fold lower than the $\text{brain}_{\text{ECF}}$-to-CSF concentration ratio observed in healthy rats, being most likely caused by an overestimation of the $\text{brain}_{\text{ECF}}$-to-plasma clearance rate. It indicates that under disease conditions there is a decreased active efflux from the $\text{brain}_{\text{ECF}}$ to plasma (results not shown). On the other hand, simulations indicated that the underestimation of CSF concentrations in disease conditions is most likely caused by an overestimation of the CSF flow. This is in line with the observation that several adult patients had an obstruction to normal CSF flow (Glantz et al., 1998) (results not shown).

Overall, the observed and SBPK model predicted plasma, $\text{brain}_{\text{ECF}}$, $\text{CSF}_{\text{LV}}$, $\text{CSF}_{\text{CM}}$ and $\text{CSF}_{\text{SAS}}$ concentration-time profiles in healthy and diseased rats, dogs, children and adults are summarized in figure 7.
Figure 6. (A) Observed and predicted methotrexate plasma, CSF<sub>LV</sub> and CSF<sub>SAS</sub> concentrations in (diseased) children; (B) observed and SBPK model predicted methotrexate plasma, brain<sub>ECF</sub> and CSF<sub>LV</sub> concentrations in (diseased) adults. Plasma data from adults and children were collected from different studies (see text and table 4). Adult brain<sub>ECF</sub> data were obtained from 2 patients with supratentorial glioma (Blakeley et al., 2009). Adult CSF<sub>LV</sub> data were obtained from 21 patients with meningeal leukemia or meningeal carcinomatosis (Shapiro et al., 1975) or 16 patients with primary tumor (Glantz et al., 1998). Child CSF<sub>LV</sub> data were obtained from 4 patients (serial sampling) with medulloblastoma or ependymoblastoma (Chatelut et al., 1991). Child CSF<sub>SAS</sub> data were obtained from 29 patients (single sample) with non-Hodgkin’s lymphoma (Vassal et al., 1990). The dots represent the individual data points and the gray area represents the 90% prediction confidence interval. The different boxes represent the plasma, brain<sub>ECF</sub>, CSF<sub>LV</sub> and CSF<sub>SAS</sub> data. The hepatic clearance was assumed to be equal to the renal clearance for the final SBPK predictions.

DISCUSSION

By using the parallel microdialysis probes approach, we have previously shown that, even for acetaminophen, a model compound for passive transport into, within and out of the brain, differences exist between CSF and brain<sub>ECF</sub> kinetics (Chapter 3). Furthermore, we have also shown that for quinidine, a model compound for P-gp mediated transport, differences exist between CSF and brain<sub>ECF</sub> kinetics, which are very much dependent of P-gp functionality (Chapter 4). With methotrexate being a substrate for a wide variety of transporters that are all located at the BBB and BCSFB, including RFC1 (Hinken et al., 2011), BCRP (Breedveld et al., 2007), MRP 2, 3 and 4 (Vlaming et al., 2011), OAT 1 and 3 (Takeda et al., 2002), and OATP A (Badagnani et
Figure 7. Observed and SBPK model predicted methotrexate plasma, brain ECF, CSF LV, CSF CM, and CSF SAS concentration-time profiles in (A) healthy rats (this study); (B) healthy rats (De Lange et al., 1995); (C) diseased rats (De Lange et al., 1995); (D) healthy dogs (Neuwelt et al., 1985); (E) diseased children (multiple references); and (F) diseased adults (multiple references). The dots represent the individual data points and the gray area represents the 90% prediction confidence interval. The different boxes represent the plasma, brain ECF, CSF LV, CSF CM, and CSF SAS concentrations.
al., 2006) and OATP B (van de Steeg et al., 2009), this could have major implications for the predictability of brain<sub>ECF</sub> methotrexate concentrations on the basis of CSF methotrexate concentrations.

In this study, the parallel microdialysis probes approach was used to investigate methotrexate distribution to and within the brain, and the specific contribution of the various transporters on the brain distribution of methotrexate. Probenecid was co-administered as inhibitor of Mrps (Bakos et al., 2000), Oats (Sugiyama et al., 2001) and Oatps (Kis et al., 2013). Probenecid is known to inhibit Mrps, Oats and Oatps with a half-maximum inhibition constant (IC50) of approximately 50-300 µg/ml (Bakos et al., 2000), 5-20 µg/ml (Sugiyama et al., 2001) and 1.1 µg/ml (Kis et al., 2013), respectively. Previous work by Emanuelsson and Paalzow (1988) has indicated that a 150 mg/kg dose of probenecid results in plasma concentrations over 10-fold higher than the IC<sub>50</sub> value up to 3 h after administration. Therefore, it is plausible to assume that the dose of probenecid is sufficient to fully inhibit Mrps, Oats and Oatps throughout the entire experimental period.

We investigated the direct relationships between brain ST methotrexate concentrations and those in different CSF locations, and unbound plasma methotrexate concentrations in the rat. Previous work from our group has indicated that the methotrexate brain<sub>ECF</sub>-to-plasma<sub>total</sub> AUC ratio is approximately 5% in healthy rats (De Lange et al., 1995). Similar findings have been reported for the CSF-to-plasma<sub>total</sub> AUC ratio (Wang et al., 2003). When taking into consideration the level of plasma protein binding and the differences in sampling methods, our current results are in line with these findings. Combined results of De Lange et al. (1995), and Wang et al. (2003), would indicate that brain<sub>ECF</sub> exposure and CSF exposure are more-or-less similar. However, in the current study, measuring parallelly at both sites within individual rats, we have found that brain<sub>ECF</sub> exposure of methotrexate is significantly higher than CSF exposure. Inhibition of Mrps, Oats and Oatps by probenecid resulted in a significant increase in brain<sub>ECF</sub> concentrations only. CSF concentrations seemed to be affected, but not to a significant extent, due to variability (as expected for a drug like methotrexate (Spector and Johanson, 2010)). Interestingly, the brain<sub>ECF</sub>-to-CSF concentration ratio was not significantly influenced by co-administration of probenecid. This is in contrast
with the P-gp substrate quinidine, for which the relation between brain_{ECF} and CSF concentrations was dependent on P-gp functionality (Chapter 4).

Advanced mathematical modeling was applied, using the same structural SBPK model that was previously used for acetaminophen (Chapter 3) and quinidine (Chapter 4). For methotrexate the model parameters were estimated using the experimental data obtained in this study. The resulting SBPK model was further used to predict data obtained in other conditions and species, taking into account changes in physiological parameters.

First, the SBPK model was used to investigate the impact of disease-status on the PK of methotrexate in plasma_u and brain_{ECF} of brain tumor-bearing rats, compared to healthy control rats as presented by De Lange et al. (1995). Figure 4A shows that the model prediction of plasma and brain_{ECF} concentrations is reasonably good. Then, figure 4B shows the SBPK prediction for healthy rat conditions, for rats in which a tumor (rhabdomyosarcoma) had been implanted in the brain (De Lange et al., 1995). It shows that tumor-bearing rats have specifically increased early methotrexate brain_{ECF} concentrations.

Then, systems-based scaling of our healthy rat data to healthy dogs initially resulted in a 10-to-100-fold underestimation of plasma_u and CSF concentrations. However, by taking into account that hepatic elimination of methotrexate in dogs is only a fraction of the renal clearance (Henderson et al., 1965), the prediction of plasma_u and CSF concentrations is much improved. The CSF_{CM}-to-plasma_u concentration ratio was predicted correctly. This implicates that the SBPK model of brain distribution developed for the healthy rat seemed to apply correctly for healthy dogs, provided that the hepatic clearance is corrected. Our ultimate aim is to predict human brain distribution, in health and disease. The human methotrexate data available in literature, however, were all obtained under disease conditions, hampering a direct evaluation of the SBPK model in predicting human brain distribution of methotrexate. But, with the assumption that the SBPK model could appropriately predict methotrexate brain distribution in humans, the SBPK model can be used to identify changes brought about by disease conditions. To that end, it should be realized that not only brain diseases can influence brain distribution, as was demonstrated by changes in BBB transport of the permeability marker fluorescein for rats with peripheral inflammation upon chronic exposure to rotenone (Ravenstijn et al., 2008).
Extrapolation of our healthy rat data to humans with different disease states initially resulted in a 100-fold underestimation of plasma_{\text{u}}, brain_{\text{ECF}} and CSF concentrations. However, by taking into account that methotrexate undergoes extensive enterohepatic circulation in humans (Hendel and Brodthagen, 1984), the prediction of plasma_{\text{u}} concentrations is much improved. Yet, under the given disease conditions, the brain_{\text{ECF}} and CSF concentrations are up to 10-fold higher than predicted for healthy conditions. Using the SBPK model, simulations indicate a possible decreased active efflux from the brain_{\text{ECF}} as well as a lower CSF flow under disease conditions. Actually, Glantz et al. (1998) reported that several patients had abnormal (low) CSF flow. It should be realized however, that the human data available from literature reflect methotrexate disposition in body and brain in a variety of diseases that probably do not affect body processes in the same manner. So, more specific data are needed to identify specific disease-related processes that influence brain distribution of methotrexate, and could lead to more personalized treatment.

Alternatively, or in addition, differences between SBPK predicted human healthy and the observed human disease methotrexate data might originate from influences of co-medication, sampling methods and analysis methods (table 4).

Possible species differences in the abundance levels and activities of the different active transport proteins at the BBB and BCSFB, under healthy and diseased conditions, probably also play an important role. It has been previously shown that the genetic variability in transporters in humans leads to an altered sensitivity to methotrexate and thus influences the toxicity and/or efficacy of methotrexate treatment (Kotnik et al., 2011). This indicates that additional information on the species differences in abundance levels and activities of the different active transport proteins and drug-metabolizing enzymes at the BBB and BCSFB, as well as at the liver and kidney, under healthy or diseased conditions, is essential for extrapolation purposes.
Table 4. Different variables in disease states and experimental conditions of the available datasets in rats, dogs, children and adults

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Disease state</th>
<th>Dose (i.v.)</th>
<th>Brain_ECF or CSF (sampling method)</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>This manuscript</td>
<td>Rats (225-275 g; n=32)</td>
<td>Healthy</td>
<td>40 and 80 mg/kg (10 min)</td>
<td>Brain_ECF, CSF_LV and CSF_CM (microdialysis)</td>
<td>LC/MS/MS</td>
</tr>
<tr>
<td>De Lange et al., 1995</td>
<td>Rats (160-200 g; n=12)</td>
<td>Brain tumor (implanted rhabdomyosarcoma)</td>
<td>75 mg/kg (bolus)</td>
<td>Brain_ECF (microdialysis)</td>
<td>HPLC</td>
</tr>
<tr>
<td>Neuwelt et al., 1985</td>
<td>Dogs (20-25 kg; n=4)</td>
<td>Healthy</td>
<td>4 mg/kg (bolus) + Evans blue</td>
<td>CSF_CM (Cisterna magna sampling)</td>
<td>Radioimmuno-assay</td>
</tr>
<tr>
<td>Aumente et al., 2006</td>
<td>Children (0.5-17y; n=49)</td>
<td>Acute lymphoblastic leukemia</td>
<td>3 g/m² (4 h) + remission-induction therapy (&gt;24h after methotrexate)</td>
<td>N.A.</td>
<td>Fluorescence polarization immunoassay</td>
</tr>
<tr>
<td>Blakeley et al., 2009</td>
<td>Human adults (&gt;18y; n=2)</td>
<td>Recurrent high grade gliomas</td>
<td>12 g/m² (4h) + sodium bicarbonate</td>
<td>Brain_ECF (intratumoral microdialysis)</td>
<td>LC/MS</td>
</tr>
<tr>
<td>Bore et al., 1987</td>
<td>Human adults (17-67y; n=8)</td>
<td>Sarcoma, carcinoma, lung metastasis</td>
<td>50 mg/m² (bolus) (no co-medication)</td>
<td>N.A.</td>
<td>Radioimmuno-assay</td>
</tr>
<tr>
<td>Chatelut et al., 1991</td>
<td>Children (2-17y; n=4)</td>
<td>Medulloblastoma or ependymoblastoma</td>
<td>8 g/m² (4 h) + sodium bicarbonate (i.v. hydration)</td>
<td>CSF_LV (ventriculoperitoneal derivation)</td>
<td>Enzymatic assay</td>
</tr>
<tr>
<td>Colom et al., 2009</td>
<td>Children (n=14)</td>
<td>Osteosarcoma</td>
<td>12 g/m² (4 h) + leucovorin (24h after methotrexate)</td>
<td>N.A.</td>
<td>Fluorescence polarization immunoassay</td>
</tr>
<tr>
<td>Creaven et al., 1976</td>
<td>Human adults (n=9)</td>
<td>Small-cell lung carcinoma</td>
<td>15 mg/m² (bolus) (no co-medication)</td>
<td>N.A.</td>
<td>[³H]methotrexate</td>
</tr>
</tbody>
</table>
CONCLUSION

It is concluded that in parallel obtained data on unbound brain_{ECF}, CSF and plasma concentrations, under dynamic conditions, combined with advanced mathematical modeling is a most valid approach to develop SBPK models that allow revealing the mechanisms underlying the relationship between brain_{ECF} and CSF concentrations. In contrast to the P-gp substrate quinidine and P-gp mediated active transport, for methotrexate we have found that inhibition of Mrp/Oat/Oatp-mediated active transport processes does not significantly influence the relationship between brain_{ECF} and CSF concentrations.

Our results suggest that the extrapolation of our healthy rat data to healthy dogs works reasonably well, provided that information on the different elimination routes, or the lack thereof, is included in the systems-based scaling approach. For the correct prediction of plasma_u, brain_{ECF} or CSF concentrations in diseased humans, additional information is needed on specific disease states in order to identify which processes are influenced by the disease condition to improve personalized treatment, in which the SBPK model is anticipated to be a useful tool.
APPENDIX

DIFFERENTIAL EQUATIONS

The mass balance equations describing the final SBPK model were expressed as follows:

Plasma:

\[
\frac{dA_{PL,u}}{dt} = \text{dose} - k_{PL-PER,\text{rapid}} \times A_{PL,u} + k_{PER,\text{rapid}-PL} \times A_{PER,\text{rapid}} \\
- k_{PL-PER,\text{slow}} \times A_{PL,u} + k_{PER,\text{slow}-PL} \times A_{PER,\text{slow}} - k_{PL-ECF} \times A_{PL,u} \\
+ k_{ECF-PL} \times A_{ECF} - k_{PL-LV} \times A_{PL,u} + k_{LV-PL} \times A_{LV} - k_{PL-TFV} \times A_{PL,u} \\
+ k_{TFV-PL} \times A_{TFV} - k_{PL-CM} \times A_{PL,u} + k_{CM-PL} \times A_{CM} + \left(\frac{Q_{CSF}}{V_{SAS}}\right) \times A_{SAS} - k_{E} \times A_{PL,u}
\]

\[
C_{PL,u} = \frac{A_{PL,u}}{V_{PL}}
\]

Periphery:

\[
\frac{dA_{PER,\text{rapid}}}{dt} = k_{PL-PER,\text{rapid}} \times A_{PL,u} - k_{PER,\text{rapid}-PL} \times A_{PER,\text{rapid}}
\]

\[
C_{PER,\text{rapid}} = \frac{A_{PER,\text{rapid}}}{V_{PER,\text{rapid}}}
\]

\[
\frac{dA_{PER,\text{slow}}}{dt} = k_{PL-PER,\text{slow}} \times A_{PL,u} - k_{PER,\text{slow}-PL} \times A_{PER,\text{slow}}
\]

\[
C_{PER,\text{slow}} = \frac{A_{PER,\text{slow}}}{V_{PER,\text{slow}}}
\]
Brain $ECF$:

\[
\frac{dA_{ECF}}{dt} = k_{PL-ECF} \times A_{PL,u} - k_{ECF-PL} \times A_{ECF} - \left( \frac{Q_{ECF}}{V_{ECF}} \right) \times A_{ECF}
\]

\[
C_{ECF} = \frac{A_{ECF}}{V_{ECF}}
\]

CSF $LV$:

\[
\frac{dA_{LV}}{dt} = k_{PL-LV} \times A_{PL,u} - k_{LV-PL} \times A_{LV} + \left( \frac{Q_{ECF}}{V_{ECF}} \right) \times A_{ECF} - \left( \frac{Q_{CSF}}{V_{LV}} \right) \times A_{LV}
\]

\[
C_{LV} = \frac{A_{LV}}{V_{LV}}
\]

CSF $TFV$:

\[
\frac{dA_{TFV}}{dt} = k_{PL-TFV} \times A_{PL,u} - k_{TFV-PL} \times A_{TFV} + \left( \frac{Q_{CSF}}{V_{TFV}} \right) \times A_{TFV} - \left( \frac{Q_{CSF}}{V_{TFV}} \right) \times A_{TFV}
\]

\[
C_{TFV} = \frac{A_{TFV}}{V_{TFV}}
\]

CSF $CM$:

\[
\frac{dA_{CM}}{dt} = k_{PL-CM} \times A_{PL,u} - k_{CM-PL} \times A_{CM} + \left( \frac{Q_{CSF}}{V_{TFV}} \right) \times A_{TFV} - \left( \frac{Q_{CSF}}{V_{CM}} \right) \times A_{CM}
\]

\[
C_{CM} = \frac{A_{CM}}{V_{CM}}
\]

CSF $SAS$:

\[
\frac{dA_{SAS}}{dt} = \left( \frac{Q_{CSF}}{V_{CM}} \right) \times A_{CM} - \left( \frac{Q_{CSF}}{V_{SAS}} \right) \times A_{SAS}
\]

\[
C_{SAS} = \frac{A_{SAS}}{V_{SAS}}
\]
Where:

\[ k_E = \left( CL_{E,p} + CL_{E,a} \right)/V_{PL} \]

\[ k_{PL\rightarrow PER,rapid} = CL_{PL\rightarrow PER,rapid}/V_{PL} \]

\[ k_{PER,rapid\rightarrow PL} = CL_{PL\rightarrow PER,rapid}/V_{PER,rapid} \]

\[ k_{PL\rightarrow PER,slow} = CL_{PL\rightarrow PER,slow}/V_{PL} \]

\[ k_{PER,slow\rightarrow PL} = CL_{PL\rightarrow PER,slow}/V_{PER,slow} \]

\[ k_{PL\rightarrow ECF} = (CL_{PL\rightarrow ECF,p} - CL_{PL\rightarrow ECF,a})/V_{PL} \]

\[ k_{ECF\rightarrow PL} = (CL_{ECF\rightarrow PL,p} + CL_{ECF\rightarrow PL,a})/V_{ECF} \]

\[ k_{PL\rightarrow LV} = (CL_{PL\rightarrow LV,p} - CL_{PL\rightarrow LV,a})/V_{PL} \]

\[ k_{LV\rightarrow PL} = (CL_{LV\rightarrow PL,p} + CL_{LV\rightarrow PL,a})/V_{LV} \]

\[ k_{PL\rightarrow TFV} = (CL_{PL\rightarrow TFV,p} - CL_{PL\rightarrow TFV,a})/V_{PL} \]

\[ k_{TFV\rightarrow PL} = (CL_{TFV\rightarrow PL,p} + CL_{TFV\rightarrow PL,a})/V_{TFV} \]

\[ k_{PL\rightarrow CM} = (CL_{PL\rightarrow CM,p} - CL_{PL\rightarrow CM,a})/V_{PL} \]

\[ k_{CM\rightarrow PL} = (CL_{CM\rightarrow PL,p} + CL_{CM\rightarrow PL,a})/V_{CM} \]

**NOMENCLATURE**

- \( A_i \): Amount of methotrexate in compartment i (ng)
- \( C_i \): Concentration of methotrexate in compartment i (ng/ml)
- \( k \): rate constant (min\(^{-1}\))
- \( Q \): flow rate (ml/min)
- \( CL \): clearance (ml/min)
- \( V \): volume (ml)

**Subscripts**

- \( PL \): plasma
- \( PL,u \): unbound methotrexate in plasma
- \( PER,rapid \): rapidly equilibrating peripheral compartment
- \( PER,slow \): slowly equilibrating peripheral compartment
- \( ECF \): brain\(_{ECF}\)
CSF CSF
LV lateral ventricle
TFV third and fourth ventricle
CM cisterna magna
SAS subarachnoid space

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